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DNA Single Strand Conformation Polymorphism Identifies Five Defined Strains of Hepatitis B Virus (HBV) During an Outbreak of HBV Infection in an Oncology Unit

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An outbreak of hepatitis B virus (HBV) infection in a children's oncology unit was identified in which 61 children were shown to have been infected, 59 of them asymptomatically. In order to establish whether intra-unit cross infection had occurred, we used the single strand conformational polymorphism (SSCP) technique to analyse viral isolates from 57 of the infected children and 40 unrelated controls. HBV-specific primers were designed to amplify a 189 bp fragment of DNA encompassing part of the hypervariable pre-S1 region of the HBV genome. Denatured PCR products were compared after electrophoresis through polyacrylamide gels and staining with silver. By SSCP analysis, the unrelated infections each yielded a unique electrophoretic banding pattern, indicative of a variety of distinct virus strains. In contrast, most of the oncology patients had been infected with one of only five different strains. Three major groups comprising 19, 16, and 9 patients, respectively, and two minor groups of 5 and 3 patients were identified. Results indicate the occurrence of multiple episodes of cross infection, and demonstrate the sensitivity and value of SSCP as a technique to establish common sources of infection.

KEY WORDS: silver stain, PCR primers, ssDNA, immunotolerance, common source, HBsAg, nosocomial

INTRODUCTION

Patients who are receiving treatment for haematological malignancies are at risk of becoming infected with hepatitis B virus (HBV) and numerous outbreaks, presumed to have a common source, have been described in oncology units [reviewed by Repp et al., 1993a; Tedder et al., 1995]. In June 1994, two children who were attending an oncology unit at a children's hospital in Cape Town, South Africa, developed clinical hepatitis B virus infection and one of them died of fulminant hepatitis. As a consequence all the children in the unit were screened for evidence of HBV infection. A total of 66 children were identified as hepatitis B surface antigen (HBsAg) positive of whom 5 had been identified as carriers by routine screening at the time of their first admission. The other 61 were infected subsequent to the initiation of treatment, and thus the possibility of nosocomial transmission needed verification.

To confirm a common source infection, it is necessary to demonstrate that patients have been infected with the same strain of a virus. HBV has been classified into 8 serotypes [Robinson, 1990] and 6 genotypes, based on sequence analysis of the HBs antigen open reading frame [Norder et al., 1994]. These subtypes show a typical geographical distribution, but within a particular geographical area, there may be considerable strain variation within a single subtype. At present, demonstration of relatedness at the genomic level is the most convincing evidence for nosocomial transmission. The genomes of a number of HBV isolates from around the world have been sequenced, and it appears that while certain regions of the viral genome are highly conserved, there is considerable variation in the pre-S1 and the pre-core genes of HBV, even within a single subtype [Carman et al., 1993; Feitelson, 1994]. Thus, sequence similarity in these two genes from different isolates is a strong indication of a common source of infection [Uy et al., 1992; Yusof et al., 1994].

Genome sequence analysis has been facilitated by the use of the polymerase chain reaction (PCR), but sequencing of isolates is time consuming and laborious. For this reason new PCR-based techniques, such as multiplex PCR [Repp et al., 1993b] and single stranded conforma-

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tional polymorphism (SSCP) [Yusof et al., 1994] have been used to investigate outbreaks of HBV infection. SSCP is a relatively simple method of identifying minor differences in nucleotide sequences [Rolfs et al., 1992]: a DNA sequence of interest is amplified by PCR and the PCR product is then heat denatured and subjected to gel electrophoresis, under non-denaturing conditions. Because single stranded DNA (ssDNA) assumes a folded secondary structure which is a unique function of its nucleotide sequence, fragments which have different sequences migrate at different rates through the gel. Under optimal conditions, a difference in even a single base may result in a detectable shift in mobility and SSCP has been used successfully to detect point mutations in genes [Orita et al., 1989; Kurvinen et al., 1995]. We report on the use of SSCP analysis of PCR products from the pre-S1 region of the HBV genome, to establish patterns of nosocomial transmission of HBV infection in an oncology unit.

MATERIALS AND METHODS

Patients and Controls

All children attending the oncology unit were tested for the presence of HBsAg and specific antibody (anti-HBs), and sera from 60 of the 66 children who were HBsAg positive, as well as 40 HBV-infected control patients, were analysed by SSCP. The control patients were unrelated and had been referred from a variety of different clinics. It was therefore assumed that they had not been infected from a common source.

Serology

Sera were tested for the presence of HBsAg and anti-HBs by radio-immune assay (AUSRIA, Abbott Laboratories) and those who were HBsAg positive were tested for HBeAg and anti-HBe by microparticle enzyme immunoassay (IMx, Abbott Laboratories).

Primer Design

It is not known which serotypes of HBV are most prevalent in Cape Town. Therefore degenerate primers were designed which would be able to amplify any of the 19 known strains of HBV presently registered in the gene bank: This was achieved by aligning all the complete HBV genome sequences, using the computer programme Clustal V which aligns similar nucleotide sequences according to the "best fit." The following primers, which bind to a region in the pre-S1 gene of HBV, were selected:

FPO 5′-TGGGAACAAGAG/$_{T}$CTAC-3′
RP4 5′-TCCTG/$_{A}$ACTGG/$_{C}$CGATTGGT-3′
FP7 5′-AATCCA/$_{C}$GATTGGGACT/$_{C}$TCAA-3′.

Primers FP0 and RP4 delineate a fragment of 325 bp; the third primer, FP7, was designed to use with primer RP4, in a semi-nested PCR, to generate a fragment of 189 bp. Apart from the highly conserved primer binding sites, there is considerable variation in this 189 bp region.

DNA Extraction and PCR

DNA was extracted from HBsAg positive sera, using the method described by Carman et al. [1991]. Briefly, 100 μl of serum was added to a solution containing 20 μl of 250 mM sodium acetate and 25 mmol EDTA, 95 μl of water, 15 μl of 6% sodium dodecyl sulphate, 1 μl tRNA and 30 μl proteinase K (Boerhinger Mannheim) at a concentration of 10 mg/ml. After digestion at 37°C for 18 hours, the samples were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated for 4 hours at −70°C, in the presence of 300 mmol sodium acetate, pH 4.5, and 2 volumes of absolute ethanol. After centrifugation, the DNA was washed twice with 70% ethanol, the pellet was dried and resuspended in 30 μl of water.

A 189 bp fragment from the pre-S1 region of the HBV genome was amplified in a semi-nested PCR. The initial amplification was performed in a 50 μl reaction volume, containing 1 unit of *Taq* DNA polymerase (Boerhinger Mannheim), 200 μmol of each dNTP, *Taq* polymerase buffer, and 10 pmol of each of primers FP0 and RP4. After the addition of 2 μl of DNA to the PCR master-mix, the sample was denatured at 94°C for 60 seconds. This was followed by 35 cycles of PCR, consisting of denaturation at 92.5°C for 20 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds. After the last cycle, there was a prolonged extension step at 72°C for 420 seconds. For the nested reaction, 2 μl of the outer PCR product was transferred into a fresh master-mix, containing 40 pmol each of primer RP4 and a new primer FP7. A further 35 cycles of amplification were performed in the same manner, except that the temperature of the primer annealing step was increased to 60°C.

Finally, 5 μl of PCR product were subjected to electrophoresis on 8% polyacrylamide gels and the DNA was visualized by silver staining, which was modified from the method described by Sammons et al. [1981]. Briefly, gels were fixed for 10 minutes in a solution containing 10% ethanol and 0.5% acetic acid and then agitated for 30 minutes in a solution of 0.1% silver nitrate. Gels were rinsed quickly with 4 changes of distilled water and the fixed silver ions were reduced by the addition of a solution containing 1.5% sodium hydroxide, 0.01% sodium boro-hydrate and 0.4% v/v of formalin. Development took between 10 and 30 minutes. Stained gels were stored in the solution used for fixation.

SSCP Analysis

Between 0.1 μl and 2 μl of PCR product was diluted in 10 μl of formamide, containing 0.05% bromophenol blue, and denatured at 95°C for 10 minutes. Samples were snap-cooled in a dry-ice and ethanol mixture and loaded onto 1.5 mm thick 8% polyacrylamide gels, containing 2.5% glycerol. Samples were electrophoresed at 200 volts (constant voltage) for 4 to 5 hours at 4°C. The Hoeffer Tall Mighty small vertical gel electrophoresis system was used. DNA was visualized by silver staining.

RESULTS

Serology

All children had been screened for HBsAg at the time of their first admission and 9 children were identified as HBsAg positive during the period from 1983 to 1994. At the time of the survey, in the latter part of 1994, it was found that 4 of these patients had seroconverted to anti-HBs positivity, but a further 61 children, who had originally been HBsAg negative, were found to have become HBsAg positive. All 61 were also HBeAg positive. Because testing for HBsAg after the initial screen was not common practice, it was not possible to assess the exact time of infection, but 8 of the 61 are known to have become positive for HBsAg within 6 months of first admission, and a further 13 within a year. One of these children had a short-lived asymptomatic infection and has since become anti-HBs positive.

PCR

Serum was available from 60 HBsAg positive children and PCR amplification of HBV DNA was successful in 58 of the 60. In all PCR-positive samples, a discrete band of 189 bp was visible on a silver stained polyacrylamide gel. An identical band was amplified from the DNA of all 40 HBV-infected controls.

SSCP Analysis

Two or more ssDNA bands, as well as residual undenatured double stranded DNA (dsDNA), were always seen. It was possible to distinguish between ssDNA and dsDNA by the colour of the bands after silver staining: ssDNA bands stained an orange-brown colour while the dsDNA stained a chocolate brown. An increase in the concentration of DNA in the DNA-formamide mixture, resulted in decreased efficiency of denaturation and consequently more double-stranded product was seen (Fig. 2a, lane 7). Greatest variation in the mobility of the single-stranded bands was achieved when the temperature of the gel, during electrophoresis, remained below 10°C.

Marked variation was observed in the mobility of ssDNA fragments from the 40 HBV-infected control subjects. Each individual sample produced a unique and reproducible SSCP pattern (Fig. 1). In contrast, only 6 of the 58 oncology patients yielded DNA with unique patterns, and the remaining 52 samples could be grouped on the basis of five distinctive electrophoretic patterns. Three patterns predominated: one was shared by 16 patients (pattern 1), another by 19 patients (pattern 3) (Fig. 2a,b), and the third by 9 patients (pattern 2) (Fig. 2c, lanes 4–6). In addition, two further groups, consisting of 5 and 3 patients each (patterns 5 and 4, respectively), also displayed common patterns (Fig. 2d). On the whole, each of the five patterns could easily be differentiated from the others. Pattern 2 was rather similar to pattern 1, but differences could clearly be distinguished when samples from these two groups were run together on the same gel (Fig. 2c). These findings indicate that most of the children were infected by one of 5 distinct strains of HBV and confirm that transmission was occurring in the unit.

In an attempt to identify the source of the 5 relevant strains, SSCP analysis was carried out on samples from 6 of the 9 patients who had been HBsAg positive at the time of entry into the unit. Three of these gave SSCP patterns that were unique and different from the 5 groups of hospital acquired HBV. The other three sera were all of the same pattern as that found in the largest group (pattern 3). All three of these patients had been transferred from the Eastern Cape region and were admitted during 1994 (in February, April and November, respectively). They therefore could not have been the primary source of infection in the unit, because pattern 3 was identified in sera collected from patients prior to 1994.

To show that the SSCP pattern did not vary with time within one individual, SSCP was performed on 4 specimens from one patient, taken at yearly intervals between 1992 and 1995. All four specimens showed identical electrophoretic patterns (Fig. 3).

DISCUSSION

There is a high prevalence of HBV infection in South Africa. Infection rates are highest in the rural areas where carriage rates vary between 2 and 12%, while in the cities the carriage rate is somewhat lower, estimated to be between 1 and 2% [Schoub, 1992]. Infection is frequently acquired in early childhood, but the exact mode of this transmission is unclear. The oncology unit at the Red Cross Children's Hospital in Cape Town which is situated in the Western Cape region of South Africa, attends to patients who are referred from various parts of the country, including several rural areas. These children are screened routinely at the time of admission to determine their HBV status, and during the period from 1983 to 1994, nine children were admitted who were already infected with HBV, a number which reflects the carriage rate in the general population.

It is well known that children attending units of this kind are at high risk of acquiring parenterally transmitted infections, not only because of the large number of blood transfusions that they receive, and the many invasive procedures that they undergo, but also because of the immunosuppressive side effects of their chemotherapy [Repp et al., 1993a]. Standard infection control procedures are therefore employed routinely to avoid cross infection with HBV. However, the occurrence of two cases of acute hepatitis B infection, one of them fulminant, in 1994, alerted the staff to the possibility that such transmissions were indeed occurring. A full screening program was initiated which revealed that 61 of the children currently attending the unit had been infected subsequent to their admission, and were asymptomatic carriers of HBsAg. This alarmingly high incidence provided cause for immediate concern to identify the source of infection. Family members of the children, as well as staff members in the unit, were tested and, since none of them were HBsAg positive, they were considered to be unlikely sources of infection. Confirmation

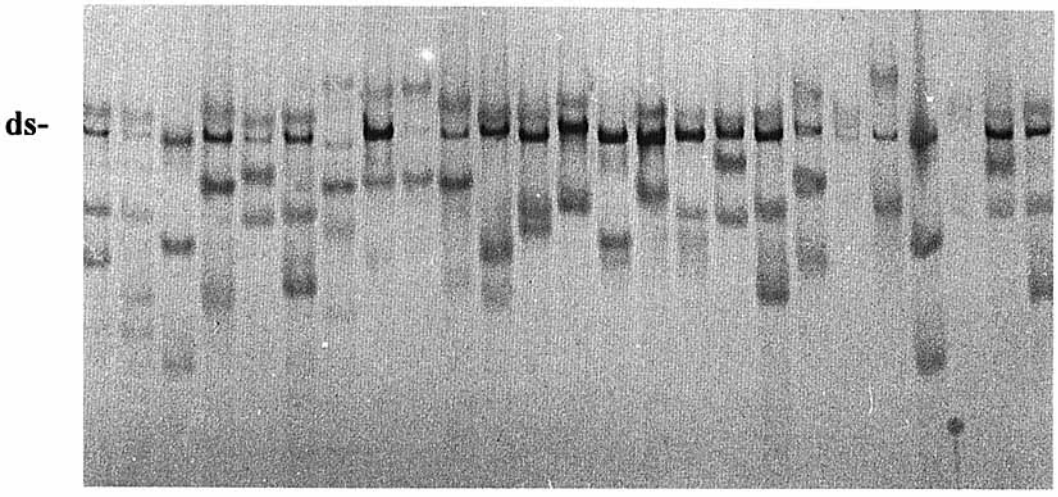

Fig. 1. SSCP electrophoretic patterns of amplified pre S1 DNA from 25 of 40 HBV-infected control patients show considerable variation. The position of the double stranded DNA band is indicated.

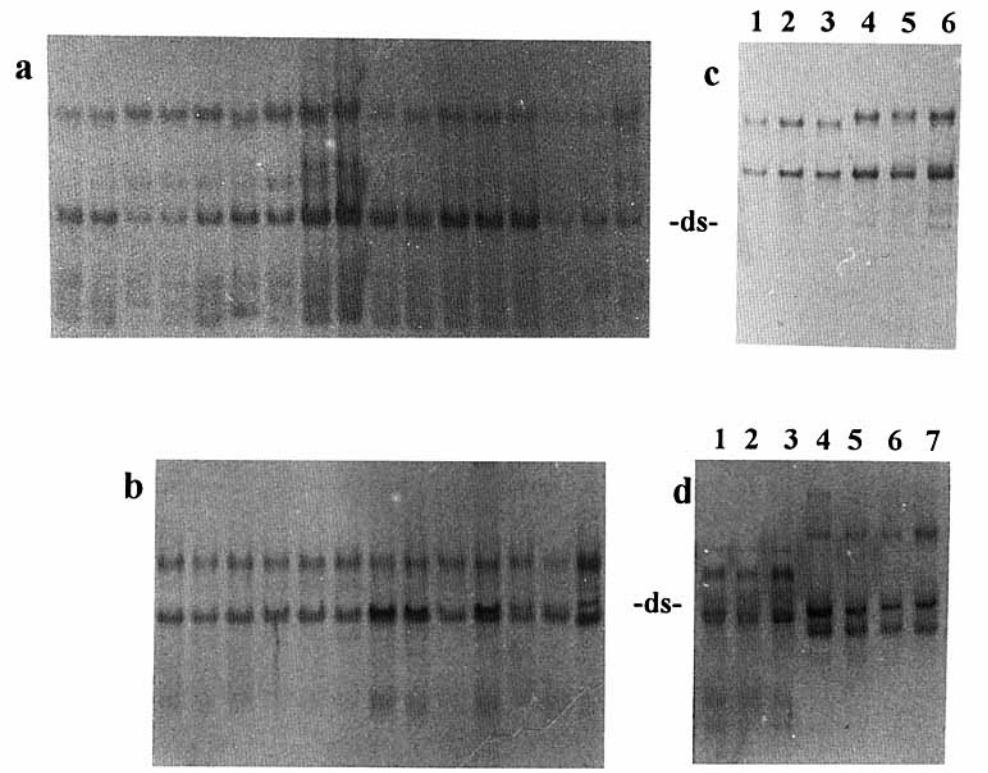

Fig. 2. Five distinct SSCP electrophoretic patterns were obtained with DNA from patients infected during the outbreak. Pattern three **(a)** and pattern one **(b),** predominated. Pattern one **(c, lanes 1–3)** and pattern two **(lanes 4–6)** were very similar, but a difference could clearly be seen when the two patterns were compared on the same gel. Patterns four, **(d, lanes 1–3)** and five **(lanes 4–7)** were detected less frequently.

of suspected nosocomial transmission was provided most convincingly by analysis of the HBV stains harboured by the infected patients. Five different strains were repeatedly identified, and all except 6 of the 58 children investigated were infected by one of these 5 strains.

The technique of SSCP analysis was chosen as the means of HBV strain identification because of the large number of patients that were involved in the outbreak. This technique has the major advantage that a large number of isolates can be processed together. The ge-

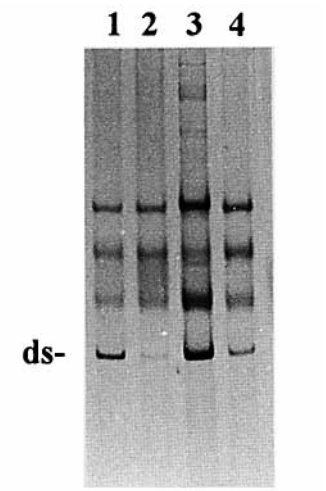

Fig. 3. SSCP patterns of 4 specimens from the same patient, taken at yearly intervals between 1992 and 1995. All four specimens show identical electrophoretic patterns.

nome sequences used for comparison should ideally cover a region that normally displays variability, and we therefore selected a 189 bp fragment from the highly variable pre-S1 gene of HBV. The length of this fragment fulfilled the criteria required for optimal separation by SSCP which is best achieved when fragment lengths are between 180 bp and 220 bp [Rolfs et al., 1992]. The pre-S1 protein is highly immunogenic and is found only in the surface of the virion, not in the excess HBsAg of the spheres and tubules. Its function is not known, but it has been postulated that it may be involved in attachment of the virion to hepatocytes [Feitelson, 1994]. The finding that randomly selected HBV-infected control patients produced a wide variety of different SSCP patterns, indicated that this pre-S1 region was a suitable target for SSCP analysis to allow clear differentiation between the local strains of HBV.

The fact that five strains of HBV were identified as being involved in cross-infections in the unit argues against a single source of infection but rather indicates a common mode of transmission. However, after careful scrutiny of all clinical procedures, the manner in which such infections had occurred could not be identified. Horizontal transmission between children remains a possibility. Most of the infected children had high titres of virus (all were HBeAg positive) so it is likely that minute amounts of blood in secretions would contain enough virus to infect highly susceptible contacts. In addition, damage to mucous membranes as a result of chemotherapy is likely to increase the contamination of saliva with blood and increase the chances of spreading the virus [Gray Davis et al., 1989; Repp et al., 1993a]. Thus, although most of the children were treated as outpatients and were admitted to the ward only intermittently for short periods, horizontal transmission might occur following relatively trivial contact. This includes the possibility of seepage from unsealed finger-prick wounds, resulting in blood contamination of shared toys and communal eating utensils.

SSCP patterns were determined on 6 of the 9 children who were already infected with HBV at the time of their first admission, but none could be positively identified as being a primary source of infection. Three of these children had unique SSCP patterns that were unlike any of the 5 specific strains implicated in the hospital-acquired infections. The other three children (all of whom had been transferred from the Eastern Cape) were all infected with the same strain of HBV (pattern 3). The pattern 3 strain was also shared by 16 other children, some of whom had been infected prior to the arrival of the three patients from the Eastern Cape who could thus not be considered as primary sources of the pattern 3 strain, although they might have contributed to the subsequent high prevalence of that strain in the unit. The most unusual discovery (by SSCP) of three patients who were infected with one and the same strain of HBV prior to admission, must be considered as significant, and strongly indicates a common source of their infections. The fact that they all originated from the same area, implies the possibility of similar cross-infections having occurred in a referring center. Alternatively, one specific strain of HBV may be characteristic of the Eastern Cape geographical area. Of note, Uy et al. [1992] have reported that a number of unrelated patients in northern Germany had an identical pre-S1 sequence. However, all of the control patients from the Western Cape displayed unique SSCP patterns (indicating a wide variety of strains in the community) and it is likely that the same situation applies to most parts of the country, except perhaps in small isolated communities.

Of the 61 children who are known to have been infected during this outbreak, only 2 had clinically apparent hepatitis, and one other child had a self-limiting asymptomatic infection. All the others have remained chronically infected, and HBeAg positive. This is consistent with the finding [Repp et al., 1993a] that patients who become infected with HBV while undergoing chemotherapy develop a state of immuno-tolerance to viral antigens, characterized by a persistently high viraemia, but there is usually minimal liver damage. Because these infections are in most instances asymptomatic, their detection, in the absence of regular laboratory screening, will tend to go unnoticed. This is particularly true if infection is acquired during chemotherapy and the state of immunotolerance persists even after cessation of therapy so that patients seldom clear the infection.

The children attending the local oncology unit were suffering from a variety of malignancies including acute leukaemias, lymphomas, as well as other solid tumours such as Wilms tumours and neuroblastomas. There was no obvious correlation between the nature of their malignancy and the risk of HBV infection, although surprisingly none of the patients with acute myeloid leukaemia (who receive most intensive chemotherapy) were shown to have been infected. It is known that the immunosuppressive effects of chemotherapy can result in the recrudescence of HBsAg antigenaemia from a previous infec-

tion that had apparently resolved. Since the majority of the infected children yielded viruses that were limited to one of only five different strains, it is unlikely that their antigenaemia could be attributed to HBV reactivation, although that mechanism may account for the positive HBsAg status of some of the five patients who displayed unique SSCP patterns.

The genomic analysis of HBV isolates from infected children has conclusively demonstrated that endemic cross infections did occur, but because no obvious mode of transmission could be identified, preventative measures are difficult to implement. As observed by others [Russo et al., 1994], active immunization appears to be of little value in the prevention of HBV transmission in these immunocompromised patients. In fact, three children from this unit who had each received three doses of HBV vaccine, subsequently became infected with HBV and all three have remained carriers. Immunization of all infants against HBV was introduced into South Africa in April 1995 and it is hoped that this will protect such children in the future from infection with HBV. However, there is naturally a concern to protect children from infection as far as possible now, and the present policy is to passively immunize all children in the unit with HBV immune globulin. It is hoped that this practice will reduce transmission of infection and, to assess its efficacy, all children will be monitored for HBsAg on a monthly basis.

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